PCT/EP2004/006157

08.06.2004

Europäisches Patentamt | European Patent Office | Office européen des brevets

REC'D 13 JUL 2004

WIPO PCT

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

03014640.1

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts; Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 03014640.1
Demande no:

Anmeldetag:
Date of filing: 26.06.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Degussa AG

40474 Düsseldorf
ALLEMAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Feedback resistant mutants

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N9/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

Feedback Resistant Mutants

The present invention is directed to specific nucleic acids and polypeptides coded by these nucleic acids as well as their application. The polypeptides of the present invention serve to improve the production of branched-chain amino acids by fermentation.
In particular, the present invention provides nucleotide sequences coding for acetohydroxy acid synthetase (AHAS) mutants, the mutated enzymes themselves and a process for the fermentative production of branched-chain amino acids using these enzymes in specific hosts in which genes which code for the modified acetohydroxy acid synthetase (AHAS) are expressed.

It is known that amino acids may be produced by fermentation of strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Due to their great significance, efforts are constantly being made to improve the production process. Improvements to the process may relate to measures concerning fermentation technology, for example stirring and oxygen supply, or to the composition of the nutrient media, such as for example sugar concentration during fermentation, or to working up of the product by, for example, ion exchange chromatography, or to the intrinsic performance characteristics of the micro-organism itself.

The performance characteristics of these microorganisms are improved using methods of mutagenesis, selection and mutant selection. In this manner, strains are obtained which are resistant to antimetabolites, such as for example the isoleucine analogue isoleucine hydroxyamate (Kisumi M, Komatsubara S, Sugiura, M, Chibata I (1972) Journal of Bacteriology 110: 761-763), the valine analogue 2-thiazolealanine (Tsuchida T, Yoshinanga F, Kubota K, Momose H (1975) Agricultural and Biological Chemistry, Japan 39: 1319-1322) or the leucine analogue α-aminobutyrates (Ambe-

Ono Y, Sato K, Totsuka K, Yoshihara Y, Nakamori S (1996) Bioscience Biotechnology Biochemistry 60: 1386-1387) or which are auxotrophic for regulatorily significant metabolites and produce e.g. branched-chain amino acids (Tsuchida T, Yoshinaga F, Kubota K, Momose H, Okumura S (1975) Agricultural and Biological Chemistry; Nakayama K, Kitada S, Kinoshita S (1961) Journal of General and Applied Microbiology, Japan 7: 52-69; Nakayama K, Kitada S, Sato Z, Kinoshita (191) Journal of General and Applied Microbiology, Japan 7: 41-51).

For some years, the methods of recombinant DNA technology have also been used for strain improvement of strains of *Corynebacterium* which produce branched-chain amino acids by amplifying individual biosynthesis genes for branched-chain amino acids and investigating the effect on their production. Review articles on this subject may be found inter alia in Kinoshita ("Glutamic Acid Bacteria", in: Biology of Industrial Microorganisms, Demain and Solomon (Eds.), Benjamin Cummings, London, UK, 1985, 115-142), Hilliger (BioTec 2, 40-44 (1991)), Eggeling (Amino Acids 6:261-272 (1994)), Jetten and Sinskey (Critical Reviews in Biotechnology 15, 73-103 (1995)), Sahm et al. (Annuals of the New York Academy of Science 782, 25-39 (1996)), and Eggeling et al., Journal of Biotechnology 56: 168-180 (1997)).

Among others the branched-chain amino acids L-isoleucine, L-valine and L-leucine are used in pharmaceutical industry, in human medicine and in animal nutrition. One of the key enzymes of the synthesis of all three amino acids in bacteria is the acetohydroxy acid synthetase (AHAS). It catalyses two reactions giving rise to precursors of the three amino acids.

In valine and leucine biosynthesis pathway, the substrate for AHAS is pyruvate. AHAS catalyses the decarboxylation of pyruvate and its condensation with the second molecule of pyruvate to produce acetolactate. In the isoleucine

pathway, AHAS catalyses reaction of pyruvate and 2-ketobutyrate producing acetohydroxy butyrate. In *Escherichia coli* strains, as much as three AHAS isoenzymes exist. Activity of the isoenzymes is inhibited by combinations of amino acids, from which the inhibition by valine is the strongest (De Felice, M., Levinthal, M., Iaccarino, M., Guardiola, J., 1979. Growth inhibition as a consequence of antagonism between related amino acids: effect of valine in Escherichia coli K12. Microbiol Rev 43, 4258). AHAS I, coded by the genes *ilvBN*, is inhibited by valine and isoleucine, AHAS II, coded by *ilvGM* is valine resistant and AHAS III, coded by *ilvIH* is inhibited by valine and isoleucine. In all cases the enzyme consists of 2 subunits. In AHAS I and AHAS III the small regulatory subunits coded by the genes *ilvN* and *ilvH* , respectively, are responsible for the inhibition.
In contrast to *E. coli*, *ilvBN* codes for the only AHAS in *C. glutamicum* (Keilhauer, C., Eggeling, L., Sahm, H., 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the ilvB-ilvN-ilvC operon. J. Bacteriol. 175, 5595-5603). Activity of the *C. glutamicum* enzyme is inhibited by valine, leucine and isoleucine (Eggeling, I., Cordes, C., Eggeling, L., Sahm, H., 1987. Regulation of acetohydroxy acid synthetase in *Corynebacterium glutamicum* during fermentation of alfa-ketobutyrate to L-isoleucine. Appl Microbiol Biotechnol 25, 346-351). Expression of the gene cluster *ilvBNC* is also regulated by these three amino acids through the transcriptional attenuation (Morbach, S., Junger, C., Sahm, H., Eggeling, L., 2000. Attenuation control of ilvBNC in *Corynebacterium glutamicum*: evidence of leader peptide formation without the presence of a ribosome binding site. J Biosci Bioeng 90, 501-507).

In *Corynebacterium glutamicum* no mutations deregulating the AHAS activity has been described on molecular level until now.

The object of the present invention was to provide a modified acetohydroxy acid synthetase (AHAS). In particular the AHAS of the present invention shall be less prone to inhibition by amino acids just produced.

This goal is meet according to the claims. Claim 1 is directed to specific nucleic acids which code for a polypeptide comprising envisaged features. Claim 2 embraces the polypeptides themselves. Claim 3 and 4 disclose hosts comprising the nucleic acids of the invention or special primers or probes for their production via PCR. Moreover, claim 5 specifies a process for the production of further improved polypeptides of the inventions, whereas claim 6 protects the thus produced polypeptides and nucleic acids, respectively. Claim 7 and 8 are directed to special uses and claim 9 embraces a process for the production of amino acids. Likewise claim 10 and 11 provide special vectors and micro-organisms.

By providing isolated nucleic acid sequences coding for a polypeptide having acetohydroxy acid synthetase (AHAS) activity selected from the group consisting of:

a) a nucleic acid sequence according to SEQ. ID No: 1 or SEQ. ID NO: 3;
b) a nucleic acid sequence comprising in position 21 and 22 a base triplet coding for Asp and Phe, respectively;
c) a nucleic acid sequence hybridising under stringent conditions with those of a) or b);
d) a nucleic acid sequence having a homology of at least 70% with those of a) or b);
e) a nucleic acid coding for a polypeptide having at least 80% homology on amino acid level with the polypeptide coded by a) or b);
f) a nucleic acid coding for a polypeptide with improved activity and/or selectivity and/or stability as compared with the polypeptide coded by a) or b), prepared by

i) mutagenesis of a nucleic acid of a) or b),
ii) ligating the nucleic acid sequence obtainable from i) into a suitable vector followed by transformation into a suitable expression system and
iii) expression and detection of the critical polypeptide with improved activity and/or selectivity and/or stability;

g) a nucleic acid sequence containing at least 15 successive bases of the nucleic acid sequences of a) - f).,

the obstacles presented above and known from the prior art have surprisingly been overcome in a notwithstandingly superior fashion. The nucleic acids of the invention encode polypeptides having a decreased amino acid feedback inhibition action compared to the wild type enzyme.

The procedure to improve the nucleic acids according to the invention or the polypeptides coded by them using the methods of mutagenesis is sufficiently well-known to a person skilled in the art. Suitable methods of mutagenesis are all the methods available for this purpose to a person skilled in the art. In particular these include saturation mutagenesis, random mutagenesis, in vitro recombination methods and site-directed mutagenesis (Eigen, M. and Gardiner, W., Evolutionary molecular engineering based on RNA replication, *Pure Appl. Chem.* **1984**, *56*, 967-978; Chen, K. and Arnold, F., Enzyme engineering for non-aqueous solvents: random mutagenesis to enhance activity of subtilisin E in polar organic media. *Bio/Technology* **1991**, *9*, 1073-1077; Horwitz, M. and Loeb, L., Promoters Selected From Random DNA-Sequences, *Proc Natl Acad Sci USA 83*, **1986**, 7405-7409; Dube, D. and L. Loeb, Mutants Generated By The Insertion Of Random Oligonucleotides Into The Active-Site Of The Beta-Lactamase Gene, *Biochemistry* **1989**, *28*, 5703-5707; Stemmer, P.C., Rapid evolution of a protein *in vitro* by DNA shuffling, *Nature* **1994**, *370*, 389-391 and Stemmer,

in the amino acid sequence of proteins from so-called superfamilies are also of use in this regard (Firestine, S. M.; Nixon, A. E.; Benkovic, S. J. (1996), Threading your way to protein function, Chem. Biol. 3, 779-783). Further information on this topic can be found in Gait, M. J. (1984), Oligonucleotide synthesis: a practical approach, IRL Press Ltd., Oxford; Innis, M. A.; Gelfound, D. H.; Sninsky, J. J. and White, T.J. (1990), PCR Protocols: A guide to methods and applications, Academic Press Inc., San Diego. The following primers are extremely preferred:

MILVNH: 5´GCGGAGGAAGTACTGCC 3´ SEQ. ID NO: 5

MILVND: 5´CAATCAGATTAATTGCTGTTTA 3´ SEQ. ID NO: 6

ILVM1: 5´GGACGTAGACGG(A)**TGA**CA(T)TTTCCCGCG 3´ SEQ. ID NO: 7

MISBGL: 5´GTTTAGA**A**CTTGGCCGGAG 3´ SEQ. ID NO: 8

SILVNH: 5´ GATCCTGCCGACATTCACGA 3´ SEQ. ID NO: 9

Such nucleic acid sequences acting as probes or primers have at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleic acids in common with those of the invention. Nucleic acid sequences having a length of at least 40 or 50 base pairs are also suitable.

A further embodiment of the present invention is directed to a process for preparing improved rec-polypeptides with acetohydroxy acid synthetase (AHAS) activity starting from nucleic acid sequences in accordance with the invention, characterised in that

a) the nucleic acid sequences are subjected to mutagenesis,

b) the nucleic acid sequences obtainable from a) are cloned in a suitable vector and these are transferred into a suitable expression system and

c) the polypeptides with improved activity and/or selectivity and/or stability which are formed are detected and isolated.

The invention also provides rec-polypeptides or nucleic acid sequences coding for these which are obtainable by a

process like the one just described.
Preparation of the nucleic acid sequences required to produce the improved rec-polypeptides and their expression in hosts is described supra and accordingly applies here.

The polypeptides and improved rec-polypeptides according to the invention are preferably used to prepare enantiomer-enriched branched-chain amino acids, more preferably valine, leucine and isoleucine.
In addition the nucleic acid sequences and improved nucleic acid sequences may preferentially be used to prepare an branched-chain amino acid producing micro-organism.

A next development of the invention reflects a process for the production of branched-chain amino acids with utilises a polypeptide of the invention.

Moreover vectors pECKA (Fig. 1) or pECKA/ilvBNC (Fig. 2) are embraced by present invention. Furthermore modified micro-organisms like DSM15652, DSM15561 or DSM15650 are enclosed in present invention. They were deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, according to the Budapest Treaty on June 04, 2003.

For cloning of the *ilvBNC* operon containing the mutations in the *ilvN* gene, the shuttle vector *Escherichia coli* – *Corynebacterium glutamicum* was constructed. First recognition site for the restriction enzyme *Bgl*II was removed from the vector pK19. Then, *Hind*III/*Hind*II fragment (2.7 kb) of the plasmid pBL1 from *Brevibacterium lactofermentum* was cloned into *Nhe*I site of pK19. The resulting plasmid vector pECKA (5.4 kb) replicates in *Escherichia coli* and *Corynebacterium glutamicum*, provides 7 unique cloning sites, kanamycin resistance marker and α-complementation of β-galactosidase for cloning in *E. coli*. The Chromosomal fragment *Ssp*I/*Eco*RI (5.7 kb) (with *Ssp*I+*Bam*HI ends) carrying the *ilvBNC* operon was cloned into

the *Hind*II+*Bam*HI-digested vector pECKA to create pECKA*ilvBNC* (11.1 kb).

The natural *Sca*I/*Bgl*II fragment of *ilvBNC* operon (770 bp) was exchanged with the same fragment containing 3 to 5 base alterations constructed by site-directed mutagenesis. The target for site-directed mutagenesis was the conserved domain of the regulatory subunit coded by *ilvN* near the N terminus. Mutations were designed by PCR according to the sequences of the *Escherichia coli* and *Streptomyces cinnamonensis* AHAS mutants. Mutations were detected by sequencing.

Plasmid DNA was isolated from *Escherichia coli* and the strain *Corynebacterium glutamicum* ATCC13032Δ*ilvN* was transformed with the plasmids pECKA*ilvBNC*(WT), pECKA*ilvBNC*(M8) and pECKA*ilvBNC*(M13). The decrease of inhibition of AHAS by branched-chain amino acids was demonstrated.

"Isolated" means separated from its natural environment.

Optically enriched (enantiomerically enriched, enantiomer enriched) compounds in the context of this invention is understood to mean the presence of >50 mol% of one optical antipode mixed with the other.

The expression nucleic acid sequences is intended to include all types of single-strand or double-strand DNA and also RNA or mixtures of the same.

An improvement in activity and/or selectivity and/or stability means, according to the invention, that the polypeptides are more active and/or more selective and are more stable under the reaction conditions used. Whereas the activity and stability of enzymes for industrial application should naturally be as high as possible, with regard to the selectivity an improvement is referred to either when either the substrate selectivity decreases or the enantioselectivity of the enzymes increases. For the

expression not substantially reduced, used in this connection, the same definition applies mutatis mutandis.

The claimed protein sequences and nucleic acid sequences also include, according to the invention, those sequences which have a homology (excluding natural degeneration) of greater than 91 %, preferably greater than 92 %, 93 % or 94 %, more preferably greater than 95 % or 96 % and particularly preferably greater than 97 %, 98 % or 99 % to one of these sequences, provided the mode of action or purpose of such a sequence is retained. The expression "homology" (or identity) as used herein can be defined by the equation $H (\%) = [1 - V/X] \times 100$, where H means homology, X is the total number of nucleobases/amino acids in the comparison sequence and V is the number of different nucleobases/amino acids in the sequence being considered with reference to the comparison sequence. In each case the expression nucleic acid sequences which code for polypeptides includes all sequences which appear to be possible, in accordance with degeneration of the genetic code.

The literature references mentioned in this document are regarded as being included within the disclosure.

<210> 4
<211> 173
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: modified AHAS

<400> 4

Met Ala Asn Ser Asp Val Thr Arg His Ile Leu Ser Val Leu Val Gln
1 5 10 15

Asp Val Asp Gly Asp Phe Ser Arg Val Ser Gly Met Phe Thr Arg Arg
20 25 30

Ala Phe Asn Leu Val Ser Leu Val Ser Ala Lys Thr Glu Thr His Gly
35 40 45

Ile Asn Arg Ile Thr Val Val Val Asp Ala Asp Glu Leu Asn Ile Glu
50 55 60

Gln Ile Thr Lys Gln Leu Asn Lys Leu Ile Pro Val Leu Lys Val Val
65 70 75 80

Arg Leu Asp Glu Glu Thr Thr Ile Ala Arg Ala Ile Met Leu Val Lys
85 90 95

Val Ser Ala Asp Ser Thr Asn Arg Pro Gln Ile Val Asp Ala Ala Asn
100 105 110

Ile Phe Arg Ala Arg Val Val Asp Val Ala Pro Asp Ser Val Val Ile
115 120 125

Glu Ser Thr Gly Thr Pro Gly Lys Leu Arg Ala Leu Leu Asp Val Met

130 135 140

Glu Pro Phe Gly Ile Arg Glu Leu Ile Gln Ser Gly Gln Ile Ala Leu
145 150 155 160

Asn Arg Gly Pro Lys Thr Met Ala Pro Ala Lys Ile
165 170

<210> 5
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Primer

<400> 5
gcggaggaag tactgcc 17

<210> 6
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Primer

<400> 6
caatcagatt aattgctgtt ta 22

<210> 7

<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Primer

<400> 7
ggacgtagac ggtgacattt cccgcg 26

<210> 8
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Primer

<400> 8
gtttagaact tggccggag 19

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Primer

<400> 9
gatcctgccg acattcacga 20

<210> 10

<211> 57

<212> PRT

<213> Corynebacterium glutamicum

<400> 10

Met Ala Asn Ser Asp Val Thr Arg His Ile Leu Ser Val Leu Val Gln
1 5 10 15

Asp Val Asp Gly Ile Ile Ser Arg Val Ser Gly Met Phe Thr Arg Arg
20 25 30

Ala Phe Asn Leu Val Ser Leu Val Ser Ala Lys Thr Glu Thr His Gly
35 40 45

Ile Asn Arg Ile Thr Val Val Val Asp
50 55

<210> 11

<211> 53

<212> PRT

<213> S. cinnamonensis

<400> 11

Met Ser Thr Lys His Thr Leu Ser Val Leu Val Glu Asn Lys Pro Gly
1 5 10 15

Val Leu Ala Arg Ile Thr Ala Leu Phe Ser Arg Arg Gly Phe Asn Ile
20 25 30

Asp Ser Leu Ala Val Gly Val Thr Glu His Pro Asp Ile Ser Arg Ile
35 40 45

Thr Ile Val Val Asn
    50

<210> 12
<211> 57
<212> PRT
<213> Escherichia Coli

<400> 12
Met Gln Asn Thr Thr His Asp Asn Val Ile Leu Glu Leu Thr Val Arg
1   5   10   15

Asn His Pro Gly Val Met Thr His Val Cys Gly Leu Phe Ala Arg Arg
            20   25   30

Ala Phe Asn Val Glu Gly Ile Leu Cys Leu Pro Ile Gln Asp Ser Asp
        35   40   45

Lys Ser His Ile Trp Leu Leu Val Asn
    50   55

Examples:

1. Construction of the plasmid vector pECKA

For cloning of the *C. glutamicum ilvBNC* operon containing the mutations in the *ilvN* gene and for its overexpression, the shuttle vector replicating in *Escherichia coli* and *Corynebacterium glutamicum* was constructed. First, recognition site for the restriction enzyme *Bgl*II was removed from the vector pK19 (Pridmore, R. D., 1987. New and versatile cloning vectors with kanamycin-resistance marker. Gene 56, 309-312). The plasmid pK19 was digested by *Bgl*II, blunt-ended by Klenow enzyme and religated. After ligation, *E. coli* DH5α cells were transformed with the ligation mixture and transformants containing the resulting plasmid pK19B were selected on agar plates containing kanamycin (20 mg/l). The removal of the *Bgl*II site in pK19B was confirmed by the treatment of the isolated plasmid molecule with *Bgl*II. (This removal has permitted later subcloning of the fragment carrying the *ilvN* gene into the newly constructed vector pECKA.) Then, *Hind*III/*Hind*II fragment (2.7 kb) of the plasmid pBL1 from *Brevibacterium lactofermentum* blunt-ended by the Klenow enzyme was cloned into the blunt-ended *Nhe*I site of pK19B. The resulting plasmid vector pECKA (5.4 kb) replicates in *Escherichia coli* and *Corynebacterium glutamicum*, provides 7 unique cloning sites (*Hin*dII, *Sal*I, *Bam*HI, *Sma*I, *Ava*I, *Kpn*I, *Sac*I) kanamycin resistance marker and α-complementation of β-galactosidase for cloning in *E. coli*. Its restriction and genetic map is shown in Fig. 1.

2. Cloning of the *ilvBNC* operon into the vector pECKA

The 5.7-kb fragment of *C. glutamicum* chromosome carrying the *ilvBNC* operon was obtained by digestion of the plasmid pKK5 (Keilhauer, C., Eggeling, L., Sahm, H., 1993. Isoleucine synthesis in Corynebacterium glutamicum: molecular analysis of the ilvB-ilvN-ilvC operon. J.

Bacteriol. 175, 5595-5603) with the restriction enzymes *Ssp*I and *Bam*HI. The fragment was ligated with the *Hind*II+*Bam*HI-digested vector pECKA and the ligation mixture was used for transformation of *E. coli* DH5α. The transformants were selected on the agar plates containing kanamycin (30 mg/l). The structure of the resulting plasmid pECKA*ilvBNC* (11.1 kb) was confirmed by restriction analysis. The restriction and genetic map of the plasmid pECKA*ilvBNC* is shown in Fig. 2.

3. Design of the oligonucleotide primer for mutagenesis of the *ilvN* gene

The known amino acid sequence of the regulatory subunit of AHAS coded by the *C. glutamicum ilvN* gene (GenBank accession number L09232) was aligned with the known amino acid sequences of regulatory subunits of AHAS from *Streptomyces cinnamonensis* (GenBank accession number AF175526) and from *Escherichia coli* (GenBank accession number AE016769, section 15 of the complete genome). Several mutations of *Escherichia coli* and *Streptomyces cinnamonensis* conferring resistance to valine were described (Vyazmensky, M., Sella, C., Barak, Z., Chipman, D. M., 1996. Isolation and characterization of subunits of acetohydroxy acid synthase isozyme III and reconstitution of the holoenzyme. Biochemistry 35, 10339-10346; Kopecký, J., Janata, J., Pospíšil, S., Felsberg, J., Spížek, J., 1999. Mutations in two distinct regions of acetolactate synthase regulatory subunit from Streptomyces cinnamonensis result in the lack of sensitivity to end-product inhibition. Biochem Biophys Res Commun 266, 162-166). In some strains displaying this phenotype, a mutation changing amino acid glycine to aspartate at position 20 (in *E. coli* sequence numbering) was found in both *E. coli* and *S. cinnamonensis* at the partially conserved domain near the N-terminus of the protein:

C. glutamicum (SEQ. ID NO:10)

MANSDVTRHILSVLVQDVDGIISRVSGMFTRRAFNLVSLVSAKTETHGINRITVVVD

S. cinnamonensis (SEQ. ID NO:11)

MS----TKHTLSVLVENKPGVLARITALFSRRGFNIDSLAVGVTEHPDISRITIVVN

E. coli (SEQ. ID NO:12)

MQNTTHDNVILELTVRNHPGVMTHVCGLFARRAFNVEGILCLPIQDSDKSHIWLLVN

We have designed a degenerated oligonucleotide primer ILVNM1 (SEQ. ID NO: 7) for site-directed mutagenesis of the *ilvN* gene of *C. glutamicum.* This primer may introduce mutations into the *ilvN* gene at the positions of the nucleotide triplets corresponding to the amino acids glycine, isoleucine and isoleucine at positions 20 to 22 in *C. glutamicum* AHAS regulatory subunit:

Primer ILVNM1 (SEQ. ID NO: 7):

```
     17  18  19  20  21  22  23  24
5´ G GAC GTA GAC GGT GAC ATT TCC CGC G 3´
                  A       T
```

The nucleotides altered, comparing to the sequence of the wild type, are shown in bold face. There are two degenerated positions, within triplets 20 and 22 (G or A and A or T, respectively).

4. Site-directed mutagenesis of the *ilvN* gene

Site-directed mutagenesis of the natural *Sca*I/*Bgl*II fragment of *C. glutamicum ilvBNC* operon (770 bp) was performed using PCR reactions and 4 oligonucleotide primers (Ito, W., Ishiguro, H., Kurosawa, Y., 1991. A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. Gene 102, 67-70).

The primers used:

MILVNH 5´GCGGAGGAAGTACTGCC 3´ (SEQ. ID NO: 6)

MILVND 5´CAATCAGATTAATTGCTGTTTA 3´ (SEQ. ID NO: 7)

ILVM1 5´GGACGTAGACGG**TGA**CATTTCCCGCG 3´ (SEQ. ID NO: 8)

A T

MISBGL 5´GTTTAGAACTTGGCCGGAG 3´ (SEQ. ID NO: 9)

First PCR: Using the primers MILVNH and MISBGL the fragment A (786 bp) with altered natural *Bgl*II site was amplified. Using the primers ILVM1 and MILVND the fragment B (491 bp) with mutations within *ilvN* gene was amplified. As a template, the plasmid pECKAilvBNC was used. The resulting DNA fragments were separated in the agarose gel, isolated and purified by precipitation.

Second PCR: Using primers MILVNH - MILVND and template fragments A + B (mixed in a molar ratio 1:1), a mixture of fragment C (803 bp) with mutation in *Bgl*II site and fragment D (803 bp) with mutations in the *ilvN* gene were amplified. This mixture was digested by *Sca*I and *Bgl*II and the resulting fragments were isolated from the agarose gel. The plasmid pECKA*ilvBNC* was digested by the same enzymes providing fragments of 766 bp and 10334 bp and the larger fragment was also isolated from the gel. The isolated fragments were mixed and ligated. The cells of *E. coli* DH5α were transformed by the ligation mixture and transformants were selected on the plates with kanamycin (30 mg/l). In this way, a natural *Sca*I/*Bcl*II chromosomal fragment (766 bp) in the plasmid pECKAilvBNC was exchanged for the same fragment in which *ilvN* can contain 3 to 5 altered nucleotides.

5. Sequencing of the mutants of *ilvN*

Plasmid DNA from the obtained *E. coli* DH5α clones was isolated and sequenced using the primer SILVNH and automatic sequencer Vistra (Amersham).

Primer SILVNH:

5′ GATCCTGCCGACATTCACGA 3′ (SEQ. ID NO: 9)

Clones with 2 different sequences within the triplets 20 to 22 were isolated:

Clones mutated in the *ilvN* gene obtained:

Mutant	DNA sequence	Amino acid position		
		20	21	22
WT	GGAATCATT	Gly	Ile	Ile
M8	GGTGACTTT	Gly	Asp	Phe
M13	GATGACTTT	Asp	Asp	Phe

The complete *ilvN* sequences of the mutants M8 and M13 are shown in Seq. 3 and 1, respectively.

6. Transformation of *Corynebacterium glutamicum*

Plasmid DNA was isolated from *Escherichia coli* and the strain *Corynebacterium glutamicum* ATCC13032 Δ*ilvN* was transformed with the plasmids pECKA*ilvBNC*(WT), pECKA*ilvBNC*(M8), pECKA*ilvBNC*(M11) and pECKA*ilvBNC*(M13) using the electroporation method (Liebl, W., Bayerl, A., Schein, B., Stillner, U., Schleifer, K. H., 1989. High efficiency electroporation of intact Corynebacterium glutamicum cells. FEMS Microbiol. Lett. 53, 299-303).

Transformants were selected on the plates with kanamycin (30 mg/l).

7. Measurements of the AHAS activity and of its inhibition by valine, leucine and isoleucine

Strains *C. glutamicum* ATCC13032 Δ*ilvN* carrying the plasmids pECKA*ilvBNC*(WT), pECKA*ilvBNC*(M8) and pECKA*ilvBNC*(M13) were used for measuring the activity of AHAS. The cells were cultivated in the minimal medium CGXII overnight, harvested by centrifugation and disrupted by sonication. After centrifugation (16000xg, 30 min) AHAS activity was measured in the cell-free extract. The spectrophotometric enzyme assay detects indirectly the reaction product acetolactate (Singh, B. K., Stidham, M. A., Shaner, D. L., 1988. Assay of acetohydroxyacid synthase. Anal Biochem 171, 173-179). The assay involves the conversion of the end product acetolactate to acetoin and the detection of acetoin via the formation of a creatine and naphthol complex.

The results of the enzyme activity measurements are shown in table 1. To test the inhibition of the enzyme by valine, leucine and isoleucine, the three amino acids (10mM) were separately added into the reaction mixture. The results are shown in table 2 and table 3, respectively.

Table 1. AHAS activity

Strain/plasmid	Specific AHAS activity (nmol acetoin min^{-1} mg^{-1} of protein)
C. glutamicum ATCC13032	33.7±10
C. glutamicum ATCC13032 Δ*ilvN*	0.43
C. glutamicum ATCC13032 Δ*ilvN* /pECKA*ilvBNC* (WT)	110±40
C. glutamicum ATCC13032 Δ*ilvN* /pECKA*ilvBNC*(M8)	31.1±0.9
C. glutamicum ATCC13032 Δ*ilvN* /pECKA*ilvBNC*(M13)	40.9±13

Table 2. Inhibition of AHAS activity

Strain/plasmid	Specific AHAS activity with 10mM amino acid (nmol acetoin min^{-1} mg^{-1} of prot.)			
	-	Val	Leu	Ile
C. glutamicum ATCC13032	33.7	16.9	20.9	21.2
C. glutamicum ATCC13032 Δ*ilvN* /pECKA*ilvBNC* WT	110	61.6	71.5	68.2
C. glutamicum ATCC13032 Δ*ilvN* /pECKA*ilvBNC*(M8)	31.1	35.1	34.8	32.7
C. glutamicum ATCC13032 Δ*ilvN* /pECKA*ilvBNC*(M13)	40.9	40.7	44.2	40.0

Table 3. Inhibition of AHAS activity in percentage

Strain/plasmid	Inhibition (10mM amino acid)		
	Val	Leu	Ile
C. glutamicum ATCC13032	50 %	38 %	37 %
C. glutamicum ATCC13032 ΔilvN /pECKAilvBNC WT	44 %	35 %	38 %
C. glutamicum ATCC13032 ΔilvN /pECKAilvBNC(M8)	0 %	0 %	0 %
C. glutamicum ATCC13032 ΔilvN/pECKAilvBNC(M13)	0 %	0 %	2.5 %

Claims:

1. Isolated nucleic acid sequence coding for a polypeptide having acetohydroxy acid synthetase (AHAS) activity selected from the group consisting of:
   a) a nucleic acid sequence according to SEQ. ID No: 1 or SEQ. ID NO: 3;
   b) a nucleic acid sequence comprising in position 21 and 22 a base triplet coding for Asp and Phe, respectively;
   c) a nucleic acid sequence hybridising under stringent conditions with those of a) or b);
   d) a nucleic acid sequence having a homology of at least 70% with those of a) or b);
   e) a nucleic acid coding for a polypeptide having at least 80% homology on amino acid level with the polypeptide coded by a) or b);
   f) a nucleic acid coding for a polypeptide with improved activity and/or selectivity and/or stability as compared with the polypeptide coded by a) or b), prepared by
      i) mutagenesis of a nucleic acid of a) or b),
      ii) ligating the nucleic acid sequence obtainable from i) into a suitable vector followed by transformation into a suitable expression system and
      iii) expression and detection of the critical polypeptide with improved activity and/or selectivity and/or stability;
   g) polynucleotide containing at least 15 successive bases of the polynucleotide sequences of a) - f).

2. A polypeptide selected from the group consisting of:
   a) a polypeptide coded by a nucleic acid sequence according to Claim 1;
   b) a polypeptide having a sequence in accordance with SEQ. ID NO: 2 or SEQ. ID NO: 4;
   c) a polypeptide which is at least 84 % homologous to

a polypeptide with SEQ. ID NO: 2 or SEQ: ID NO. 4, without the activity and/or selectivity and/or stability of the polypeptide being substantially reduced as compared with the polypeptide with SEQ. ID NO: 2 or SEQ. ID NO: 4.

3. Plasmids, vectors, micro-organisms comprising one or more nucleic acid sequences according to Claims 1.

4. Primers for preparing - by means of PCR - or hybridisation probes for detecting the nucleic acid sequences according to Claim 1.

5. A process for preparing improved rec-polypeptides with acetohydroxy acid synthetase (AHAS) activity starting from nucleic acid sequences in accordance with Claim 1,
characterised in that
a) the nucleic acid sequences are subjected to mutagenesis,
b) the nucleic acid sequences obtainable from a) are cloned in a suitable vector and these are transferred into a suitable expression system and
c) the polypeptides with improved activity and/or selectivity and/or stability which are formed are detected and isolated.

6. rec-polypeptides or nucleic acid sequences coding for these, obtainable in accordance with Claim 5.

7. The use of the polypeptides in accordance with Claim 2 or 6 to prepare enantiomer-enriched branched-chain amino acids.

8. Use of the nucleic acid sequences in accordance with Claim 1 or 6 to prepare an amino acid producing micro-organism.

9. Process for the production of a branched-chain amino acid using a polypeptide of Claim 2.

10. Vector pECKA or pECKA/ilvBNC.

11. Micro-organisms: DSM15652, DSM15651, DSM15650.

020191 AM

Abstract:

The present invention provides nucleotide sequences coding for acetohydroxy acid synthetase (AHAS) mutants, the mutated enzymes themselves and a process for the fermentative production of branched-chain amino acids using these enzymes in specific hosts in which genes which code for the modified acetohydroxy acid synthetase (AHAS) are expressed.

020191 AM

EPO - Munich
74
26. Juni 2003

Fig 1.

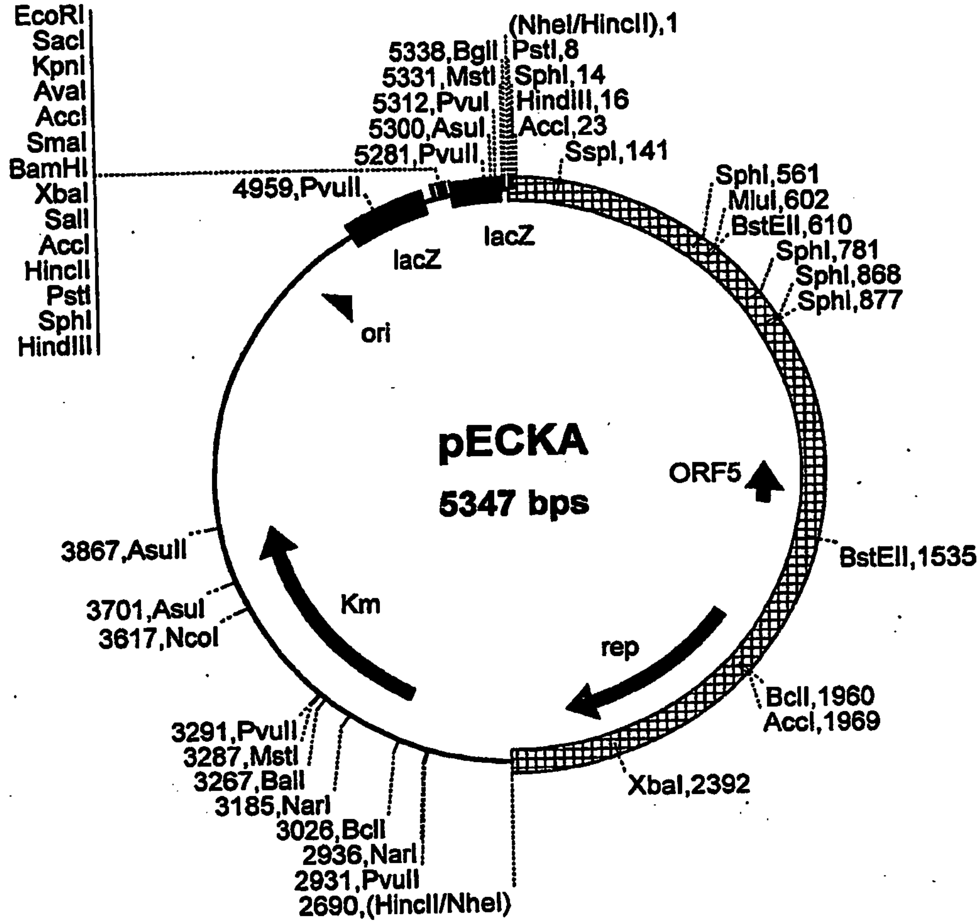
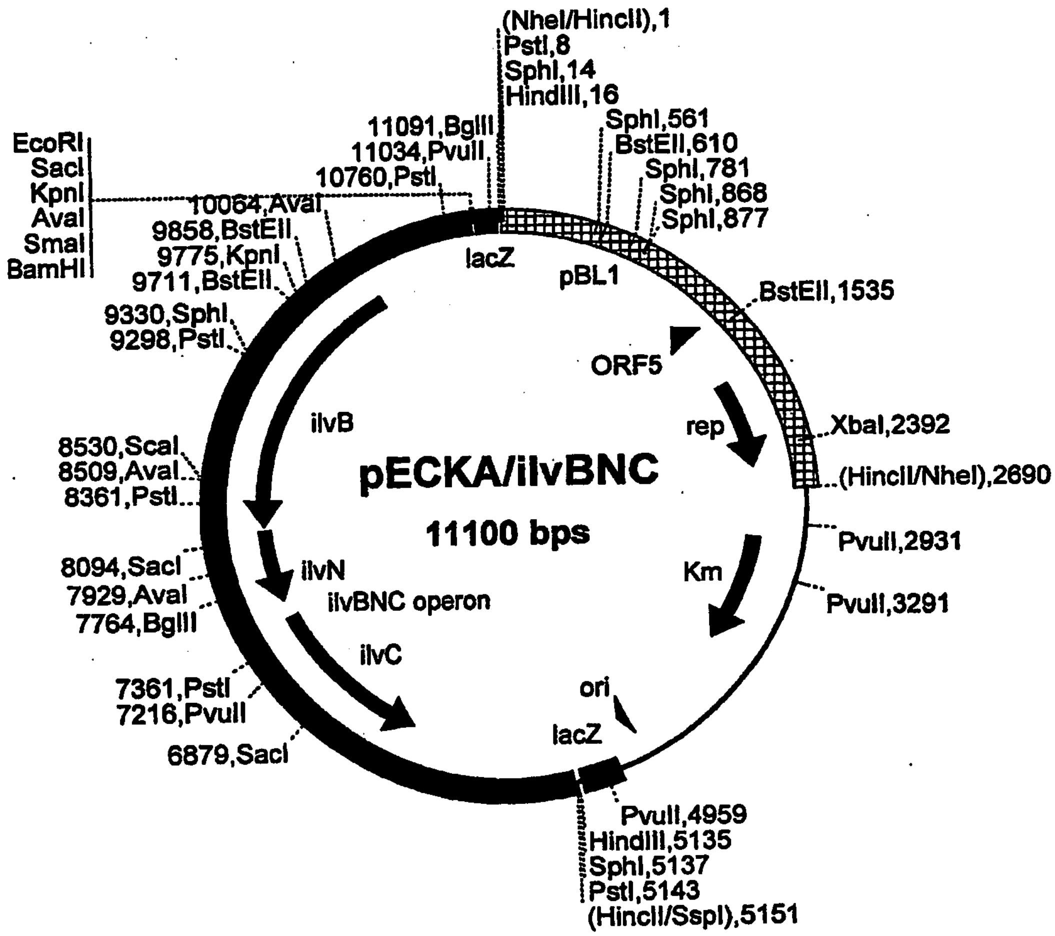


Fig. 2:


(NheI/HincII),1
PstI,8
SphI,14
HindIII,16
SphI,561
BstEII,610
SphI,781
SphI,868
SphI,877
BstEII,1535
XbaI,2392
(HincII/NheI),2690
PvuII,2931
PvuII,3291
PvuII,4959
HindIII,5135
SphI,5137
PstI,5143
(HincII/SspI),5151
6879,SacI
7216,PvuII
7361,PstI
7764,BglII
7929,AvaI
8094,SacI
8361,PstI
8509,AvaI
8530,ScaI
9298,PstI
9330,SphI
9711,BstEII
9775,KpnI
9858,BstEII
10064,AvaI
10760,PstI
11034,PvuII
11091,BglII
EcoRI
SacI
KpnI
AvaI
SmaI
BamHI
lacZ
pBL1
ORF5
rep
Km
ori
lacZ
ilvB
ilvN
ilvBNC operon
ilvC
pECKA/ilvBNC
11100 bps

SEQUENCE LISTING

<110> Degussa AG

<120> Feedback Resistant Mutants

<130> 020191 AM

<140>
<141>

<160> 12

<170> PatentIn Ver. 2.1

<210> 1
<211> 519
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: modified AHAS

<220>
<221> CDS
<222> (1)..(519)

<400> 1
atg gct aat tct gac gtc acc cgc cac atc ctg tcc gta ctc gtt cag 48
Met Ala Asn Ser Asp Val Thr Arg His Ile Leu Ser Val Leu Val Gln
1 5 10 15

gac gta gac gat gac ttt tcc cgc gta tca ggt atg ttc acc cga cgc 96
Asp Val Asp Asp Asp Phe Ser Arg Val Ser Gly Met Phe Thr Arg Arg

20 25 30

gca ttc aac ctc gtg tcc ctc gtg tct gca aag acc gaa aca cac ggc 144
Ala Phe Asn Leu Val Ser Leu Val Ser Ala Lys Thr Glu Thr His Gly
35 40 45

atc aac cgc atc acg gtt gtt gtc gac gcc gac gag ctc aac att gag 192
Ile Asn Arg Ile Thr Val Val Val Asp Ala Asp Glu Leu Asn Ile Glu
50 55 60

cag atc acc aag cag ctc aac aag ctg atc ccc gtg ctc aaa gtc gtg 240
Gln Ile Thr Lys Gln Leu Asn Lys Leu Ile Pro Val Leu Lys Val Val
65 70 75 80

cga ctt gat gaa gag acc act atc gcc cgc gca atc atg ctg gtt aag 288
Arg Leu Asp Glu Glu Thr Thr Ile Ala Arg Ala Ile Met Leu Val Lys
85 90 95

gtc tct gcg gac agc acc aac cgt ccg cag atc gtc gac gcc gcg aac 336
Val Ser Ala Asp Ser Thr Asn Arg Pro Gln Ile Val Asp Ala Ala Asn
100 105 110

atc ttc cgc gcc cga gtc gtc gac gtg gct cca gac tct gtg gtt att 384
Ile Phe Arg Ala Arg Val Val Asp Val Ala Pro Asp Ser Val Val Ile
115 120 125

gaa tcc aca ggc acc cca ggc aag ctc cgc gca ctg ctt gac gtg atg 432
Glu Ser Thr Gly Thr Pro Gly Lys Leu Arg Ala Leu Leu Asp Val Met
130 135 140

gaa cca ttc gga atc cgc gaa ctg atc caa tcc gga cag att gca ctc 480
Glu Pro Phe Gly Ile Arg Glu Leu Ile Gln Ser Gly Gln Ile Ala Leu
145 150 155 160

aac cgc ggt ccg aag acc atg gct ccg gcc aag atc taa 519

Asn Arg Gly Pro Lys Thr Met Ala Pro Ala Lys Ile

165 170

<210> 2

<211> 173

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: modified AHAS

<400> 2

Met Ala Asn Ser Asp Val Thr Arg His Ile Leu Ser Val Leu Val Gln

1 5 10 15

Asp Val Asp Asp Asp Phe Ser Arg Val Ser Gly Met Phe Thr Arg Arg

20 25 30

Ala Phe Asn Leu Val Ser Leu Val Ser Ala Lys Thr Glu Thr His Gly

35 40 45

Ile Asn Arg Ile Thr Val Val Val Asp Ala Asp Glu Leu Asn Ile Glu

50 55 60

Gln Ile Thr Lys Gln Leu Asn Lys Leu Ile Pro Val Leu Lys Val Val

65 70 75 80

Arg Leu Asp Glu Glu Thr Thr Ile Ala Arg Ala Ile Met Leu Val Lys

85 90 95

Val Ser Ala Asp Ser Thr Asn Arg Pro Gln Ile Val Asp Ala Ala Asn

100 105 110

Ile Phe Arg Ala Arg Val Val Asp Val Ala Pro Asp Ser Val Val Ile

115 120 125

Glu Ser Thr Gly Thr Pro Gly Lys Leu Arg Ala Leu Leu Asp Val Met
130 135 140

Glu Pro Phe Gly Ile Arg Glu Leu Ile Gln Ser Gly Gln Ile Ala Leu
145 150 155 160

Asn Arg Gly Pro Lys Thr Met Ala Pro Ala Lys Ile
165 170

<210> 3
<211> 519
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: modified AHAS

<220>
<221> CDS
<222> (1)..(519)

<400> 3
atg gct aat tct gac gtc acc cgc cac atc ctg tcc gta ctc gtt cag 48
Met Ala Asn Ser Asp Val Thr Arg His Ile Leu Ser Val Leu Val Gln
1 5 10 15

gac gta gac ggt gac ttt tcc cgc gta tca ggt atg ttc acc cga cgc 96
Asp Val Asp Gly Asp Phe Ser Arg Val Ser Gly Met Phe Thr Arg Arg
20 25 30

gca ttc aac ctc gtg tcc ctc gtg tct gca aag acc gaa aca cac ggc 144

Ala Phe Asn Leu Val Ser Leu Val Ser Ala Lys Thr Glu Thr His Gly
35 40 45

atc aac cgc atc acg gtt gtt gtc gac gcc gac gag ctc aac att gag 192
Ile Asn Arg Ile Thr Val Val Val Asp Ala Asp Glu Leu Asn Ile Glu
50 55 60

cag atc acc aag cag ctc aac aag ctg atc ccc gtg ctc aaa gtc gtg 240
Gln Ile Thr Lys Gln Leu Asn Lys Leu Ile Pro Val Leu Lys Val Val
65 70 75 80

cga ctt gat gaa gag acc act atc gcc cgc gca atc atg ctg gtt aag 288
Arg Leu Asp Glu Glu Thr Thr Ile Ala Arg Ala Ile Met Leu Val Lys
85 90 95

gtc tct gcg gac agc acc aac cgt ccg cag atc gtc gac gcc gcg aac 336
Val Ser Ala Asp Ser Thr Asn Arg Pro Gln Ile Val Asp Ala Ala Asn
100 105 110

atc ttc cgc gcc cga gtc gtc gac gtg gct cca gac tct gtg gtt att 384
Ile Phe Arg Ala Arg Val Val Asp Val Ala Pro Asp Ser Val Val Ile
115 120 125

gaa tcc aca ggc acc cca ggc aag ctc cgc gca ctg ctt gac gtg atg 432
Glu Ser Thr Gly Thr Pro Gly Lys Leu Arg Ala Leu Leu Asp Val Met
130 135 140

gaa cca ttc gga atc cgc gaa ctg atc caa tcc gga cag att gca ctc 480
Glu Pro Phe Gly Ile Arg Glu Leu Ile Gln Ser Gly Gln Ile Ala Leu
145 150 155 160

aac cgc ggt ccg aag acc atg gct ccg gcc aag atc taa 519
Asn Arg Gly Pro Lys Thr Met Ala Pro Ala Lys Ile
165 170